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Note

High-performance liquid chromatographic assay for aminoglutethimide and its acetylated metabolite in urine

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Aminoglutethimide (AG), 3-(4-aminophenyl)-3-ethyl piperidine-2,6-dione, is an effective agent for the treatment of advanced breast cancer in post-menopausal women [1, 2].

Metabolism studies in man have shown that, after single doses of AG, large amounts are excreted unchanged in the urine together with smaller quantities of its acetylated metabolite, N-acetyl amino-glutethimide (AcAG) [3, 4]. Some minor metabolites of the drug have also been identified as N-formyl AG and nitroglutethimide [5]. N-Hydroxyl AG has been recently described as an auto-induced metabolite that appears in the urine on chronic dosing with AG [6].

In the rat the complete metabolic fate of AG has been determined using the ^{14}C-labelled drug [7] and only small quantities of AG are excreted in urine together with larger amounts of AcAG [8].

In order to study the inter-individual variation in the excretion of AG and its acetylation profile in man and other species, we developed a simple method for the quantitative determination of AG and AcAG in urine by high-performance liquid chromatography (HPLC) using aniline as internal standard. Recently an HPLC procedure for the assay of AG in plasma has been described [9]. However, the method does not determine AcAG.

EXPERIMENTAL

Materials

Aminoglutethimide was a gift from Ciba-Geigy (Horsham, U.K.).

N-Acetyl aminoglutethimide was synthesized according to the procedure described by Douglas [10].

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Aniline hydrochloride, 99% (Aldrich, Gillingham, U.K.), was used as the internal standard.

Dichloromethane (AnalaR, BDH, Poole, U.K.) was distilled and washed with 0.1 *M* phosphate buffer pH 7.0 prior to use.

The phosphate buffers used were prepared from the AnalaR grade mono- and di-sodium phosphates using mixtures of equimolar solutions.

HPLC grade acetonitrile (Rathburn, Walkerburn, U.K.) and AnalaR grade water (BDH) were used for chromatography.

Chromatography

Chromatographic analyses were performed on a component system consisting of a Constametric II G pump, equipped with a Rheodyne 7125 syringe-loading sample injector, a Spectromonitor III ultraviolet (UV) detector and a Tekman TE 200 flatbed recorder.

Samples were chromatographed at room temperature on a Spherisorb ODS column (30 cm × 4 mm I.D., 5 μm particle size) from Jones Chromatography (Llanbradach, U.K.).

The separations were obtained using acetonitrile—0.01 *M* phosphate buffer, pH 6.8 (22:68) as the mobile phase, with a flow-rate of 1.5 ml/min. Peaks were detected at 234 nm and peak areas were measured by triangulation.

Calibration curves

From stock solutions in methanol standard solutions were prepared containing mixtures of AG (in the range 0.05—5 mg), AcAG (in the range 0.025—2.5 mg) and aniline hydrochloride (0.2 mg) per ml of methanol. Replicate injections of 10 μl were made for each sample.

Assay method

Phosphate buffer, 0.1 *M*, pH 7 (5 ml) and aqueous aniline hydrochloride solution (1 ml containing 0.1 mg) were mixed with urine (5 ml). After addition of dichloromethane (10 ml) the mixture was vortexed for 2 min. The phases were then separated by centrifuging and the aqueous layer was re-extracted with dichloromethane (10 ml). The combined organic phase was dried with anhydrous sodium sulphate, filtered and evaporated under reduced pressure at 60°C. The residue was dissolved in methanol (0.5 ml). Replicate aliquots (10 μl) were injected into the column. Earlier investigations [3, 4] have shown this extraction procedure to produce reliably high recoveries of AG and AcAG.

RESULTS AND DISCUSSION

When AG, AcAG and aniline, dissolved in methanol, were applied together to the column a good separation was obtained, the retention times (and capacity factors) being 12.6 min (4.7), 14.8 min (5.7) and 8.6 min (2.9), respectively. Solutions of the compounds in methanol over the concentration ranges 0.05—5 mg/ml AG and 0.025—2.5 mg/ml AcAG with 0.2 mg/ml aniline hydrochloride were chromatographed in this system. Linear relationships between the ratio of peak areas (AG and AcAG to internal standard) and concentration

(mg/ml) of AG and AcAG were found that could be expressed by the following equations: for AG: $Y = 2.2192\,X + 0.2168$ ($r = 0.98$); for AcAG: $Y = 1.7155\,X - 0.0730$ ($r = 0.98$) where Y represents the peak area ratio and X the concentration (mg/ml).

Fig. 1 shows chromatograms obtained from extracts of a representative sample (Fig. 1A) from a human volunteer following oral ingestion of a 250-mg tablet of AG (Orimeten, Ciba-Geigy) and of blank urine (Fig. 1B). These chromatograms demonstrate the lack of interference and the specificity of the assay procedure for the measurement of AG and AcAG in human urine.

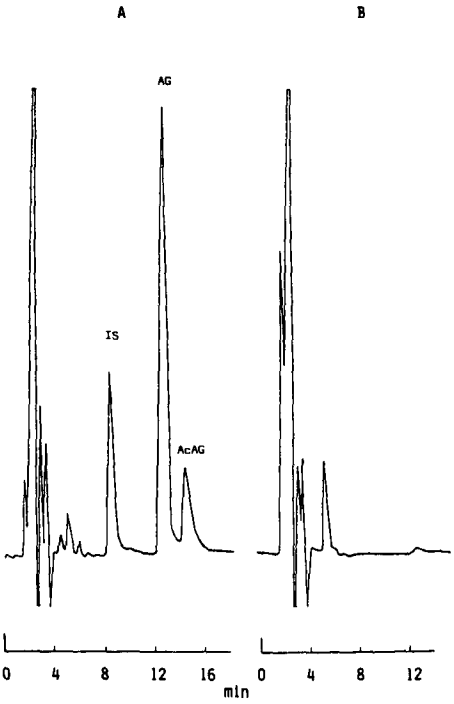

Fig. 1. Chromatograms of (A) extract of urine collected 0—24 h after the ingestion of a 250-mg tablet of aminoglutethimide; this urine contained 24.8% and 5.5% of the administered dose as AG and AcAG, respectively; (B) extract of blank human urine. UV detection: 234 nm. Peaks: IS = aniline; AG = aminoglutethimide; AcAG = N-acetyl aminoglutethimide.

Authentic samples of N-formyl AG (from AG) and nitroglutethimide (from glutethimide) were synthesized by standard methods and chromatographed in the above system. Nitroglutethimide had an elution time of 30 min, while N-formyl AG eluted between the AG and AcAG peaks. The formyl peak was never observed on the chromatograms from urine extracts of human subjects taking AG. Thus, as N-formyl AG is a minor metabolite, it was considered not to interfere with the assay of AG and AcAG. In a recent study in this laboratory, where urine extracts from patients receiving AG in various combinations with dextropropoxyphene, hydrocortisone, ibuprofen and paracetamol were chromatographed, no interfering peaks were detected.

Spiked blank human urine, containing mixtures of equal concentrations of AG and AcAG (at 10 and 20 μg/ml of urine) was carried through the ex-

traction and assay procedure. Recovery was consistent (90%) and the assay-variation (coefficient of variation, C.V., %) determined on six aliquots was 3.5% and 2.4% for AG and AcAG, respectively.

The lowest limit of detection was found to be approximately 0.2 μg in the methanolic solution injected onto the column. This is a more than adequate sensitivity for human urine in view of the relatively large doses employed in therapy and the extensive urinary excretion of the drug and its metabolites.

In a group of five male human volunteers with normal renal function receiving AG (250 mg) orally, the mean 24-h urinary excretion of unchanged drug and AcAG (as a percentage of the dose), determined by the above method, was 31.2 ± 10.1% S.D. and 10.3 ± 2.9% S.D., respectively.

The assay was also applied to the determination of AG and AcAG in urine of various animal species receiving AG (60 mg/kg) orally. Initially, representative samples of blank urine from each species were carried through the procedure. When chromatographed, the extracts presented similar elution profiles to those of blank human urine indicating the absence of interfering endogenous compounds. Following a dose of AG, peaks for AG and AcAG were detected in extracts of urine from rats, guinea pigs and rabbits. However, the extracts from these species contained additional materials (presumably other metabolites of AG) that were observed as peaks on the chromatograms. These did not interfere with the assay of AG and AcAG as they eluted prior to the internal standard. For the rat and guinea pig a further metabolite was eluted with a retention time of 17.9 min.

TABLE I

24-h URINARY EXCRETION OF AMINOGLUTETHIMIDE (AG) AND N-ACETYL AMINOGLUTETHIMIDE (AcAG) IN SEVERAL ANIMAL SPECIES AFTER AN ORAL DOSE OF AG (60 mg/kg)

Results are the means ± S.D. from four animals.

Species	Excretion (percentage of dose)	
	Ag	AcAG
Rat	1.8 ± 0.5	24.3 ± 6.2
Guinea pig	8.3 ± 1.1	4.2 ± 2.0
Rabbit	9.9 ± 4.3	8.0 ± 5.2

From the results presented in Table I it may be observed that unchanged AG was excreted in the urine of these animals to a small extent only. This was true for the excretion of AcAG in the guinea pig and rabbit. However, in the rat, AcAG appeared to be an important urinary metabolite.

In conclusion, this HPLC method is a simple, reproducible and sensitive procedure.

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